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Gluco-oligomers initially formed by the reuteransucrase enzyme of *Lactobacillus reuteri* 121 incubated with sucrose and malto-oligosaccharides

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The probiotic bacterium *Lactobacillus reuteri* 121 produces a complex, branched (1 → 4, 1 → 6)-α-D-glucan as extracellular polysaccharide (reuteran) from sucrose (Suc), using a single glucansucrase/glucosyltransferase (GTFA) enzyme (reuteransucrase). To gain insight into the reaction/product specificity of the GTFA enzyme and the mechanism of reuteran formation, incubations with Suc and/or a series of malto-oligosaccharides (MOSs) (degree of polymerization (DP2–DP6)) were followed in time. The structures of the initially formed products, isolated via high-performance anion-exchange chromatography, were analyzed by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry and 1D/2D $^{1}H/^{13}C$ NMR spectroscopy. Incubations with Suc only, acting as both donor and acceptor, resulted in elongation of Suc with glucose (Glc) units via alternating (α1 → 4) and (α1 → 6) linkages, yielding linear gluco-oligosaccharides up to at least DP ~ 12. Simultaneously with the ensemble of oligosaccharides, polymeric material was formed early on, suggesting that alternan fragments longer than DP ~ 12 have higher affinity with the GTFA enzyme and are quickly extended, yielding high-molecular-mass branched reuteran (4×10^7 Da). MOSs (DP2–DP6) in the absence of Suc turned out to be poor substrates. Incubations of GTFA with Suc plus MOSs as substrates resulted in preferential elongation of MOSs (acceptors) with Glc units from Suc (donor). This apparently reflects the higher affinity of GTFA for MOSs compared with Suc. In accordance with the GTFA specificity, most prominent products were oligosaccharides with an (α1 → 4)/(α1 → 6) alternating structure.

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Keywords: α-D-glucans / glucanotransferase / glucansucrase / *Lactobacillus reuteri* / structural analysis

Introduction

In an earlier screening of a large collection of probiotic *Lactobacillus* strains for their possible exopolysaccharide (EPS) production, we have shown that *Lactobacillus reuteri* strain 121 cells produce both a glucan (EPS121) and a fructan on sucrose (Suc) (α-D-Glc*p*-(1 ↔ 2)-β-D-Fru*f*) (Van Geel-Schutten et al. 1998). The water-soluble glucan was identified as a high-molecular-mass (3.5×10^6 Da) branched α-D-glucan with terminal, 4-substituted, 6-substituted and 4,6-disubstituted Glc units. The fructan turned out to be a low-molecular-mass (1.5×10^5 Da) (2 → 6)-β-D-fructofuranan or levan (Van Geel-Schutten et al. 1999). Interestingly, from chemostat cultures of *L. reuteri* strain 121, a spontaneous mutant *L. reuteri* strain 35-5 was isolated that lacked all levansucrase activity, but still possessed glucansucrase (glucosyltransferase (GTFA)/GTF; EC 2.4.1.5) activity and produced the wild-type α-D-glucan (EPS35-5 = EPS121) in amounts up to 10 g/L (Van Geel-Schutten et al. 1999).

Further studies with *L. reuteri* strain 121 resulted in isolation of the *gtfA* and *gtfB* genes, encoding the GTFA and GTFB enzymes, respectively (Kralj et al. 2002, 2011). The GTFA enzyme is responsible for α-D-glucan biosynthesis from Suc (Kralj et al. 2002). It belongs to glycoside hydrolase family 70 (GH70), a group of bacterial glucansucrase or GTFA enzymes (Cantarel et al. 2009). Glucansucrase enzymes differ in reaction/product specificity, synthesizing high-molecular-mass α-D-glucans with different (ratios of) glycosidic linkages and degree of branching, depending on the bacterial source of the enzyme (Kralj, van Geel-Schutten, Dondorff et al. 2004). The mechanistic and structural features determining these differences in glucansucrase reaction/product specificity are still not fully understood, and little is known about the products synthesized early in time (Monchois et al. 1999; Moulis et al. 2006; Leemhuis, Pijning et al. 2013; Leemhuis, Dijkman et al. 2013).

Also the GTFB type of enzymes is classified in family GH70. Recently, we have reported that the GTFB enzyme of *L. reuteri* strain 121 (Kralj et al. 2011; Dobruchowska et al. 2012) is inactive with Suc, but instead has hydrolysis/transferase activity on malto-oligosaccharides (MOSs) and starch-like polysaccharides (Dijkhuizen et al. 2010). The elongated linear gluco-oligomers formed contain besides (α1 → 4) a high

percentage of (α1 → 6) glycosidic linkages. In view of its reaction/product specificity, the GTFB enzyme was classified as a 4,6-α-glucanotransferase, the first example of such an enzyme activity in family GH70, representing a new subfamily (Kralj et al. 2011).

Recombinant GTFA purified from *Escherichia coli*, and the culture supernatants of *L. reuteri* strain 35-5, synthesized virtual identical α-D-glucan polymers (reuteran = EPS35-5 = EPS121) when incubated overnight with Suc (according to ^{1}H NMR analysis). Their molecular masses were determined to be 8×10^7 and 4×10^7 Da, respectively. Additionally, a detailed molecular (construction of site-directed and deletion mutants) and biochemical (main reactions catalyzed by wild-type and mutant enzymes) analysis of the GTFA enzyme, called reuteransucrase, has been reported (Kralj, van Geel-Schutten, van der Maarel et al. 2004). Recently, also the crystal structure of functional GTFA-ΔN, a 118-kDa fragment of GTFA comprising residues 745–1763 and including the catalytic domain, was determined at 3.6 Å resolution by molecular replacement (Pijning et al. 2012). A detailed structural analysis of the reuteran product formed from Suc by the recombinant glucansucrase GTFA enzyme yielded a (1 → 4, 1 → 6)-α-D-glucan, with no repeating units present. The constructed composite model is presented in Figure 1. It should be noted that the reuteran structure showed a large number of alternating (α1 → 4)/(α1 → 6) linkages (Van Leeuwen, Kralj et al. 2008).

When exploring the activity of the recombinant GTFA enzyme with the MOS series DP2–DP7, only DP4 (maltotetraose) gave some clear higher-molecular-mass bands on thin-layer chromatography (TLC) (S.K., unpublished data). However, recombinant GTFA enzyme incubated with equimolar mixtures of Suc and maltose for 60 h resulted in formation of panose (major), maltotriose (minor) and two unknown products (minor) (demonstrated by high-performance anion-exchange chromatography on CarboPac PA-1). When using equimolar amounts of Suc and isomaltose, low amounts of isomaltotriose and isomaltotetraose were seen, together with two unknown products (Kralj, van Geel-Schutten, van der Maarel et al. 2004).

To gain a better understanding of the activity of the recombinant GTFA enzyme, especially during the first hours of in vitro incubation with Suc, the present study focused specifically on the structural analysis of the initially formed oligosaccharides en route to the polysaccharide reuteran. Furthermore, product mixtures from incubation of MOS with GTFA in the absence and presence of sucrose were analyzed. In all cases, the glycan products were analyzed by high-pH anion-exchange chromatography (HPAEC), matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and 1D/2D ^{1}H/^{13}C nuclear magnetic resonance (NMR) spectroscopy, total correlation spectroscopy (TOCSY), ^{1}H detected heteronuclear single quantum coherence spectroscopy (HSQC) and rotating-frame nuclear Overhauser enhancement spectroscopy (ROESY)).

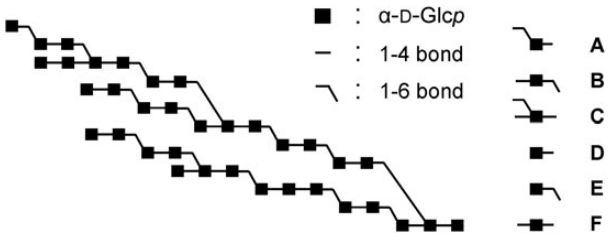

Fig. 1. Composite structure of the exopolysaccharide reuteran (EPS35-5/EPS121) of *L. reuteri* 121 obtained by incubation of Suc with recombinant GTFA enzyme (Van Leeuwen, Kralj et al. 2008).

Results

Gluco-oligosaccharides initially formed by GTFA from sucrose

To identify the initially formed oligosaccharides, precursors of reuteran, 50 mM Suc was incubated at 37°C with 50 nM recombinant GTFA in 25 mM sodium acetate buffer/1 mM $CaCl_2$, pH 4.7. The progress of the reaction in time was followed by TLC (data not shown). The formation of lower- and higher-molecular-mass products than the substrate Suc indicated hydrolysis/transferase activity, demonstrating that the GTFA enzyme is active in the absence of an exogenous primer.

The HPAEC profiles of the oligosaccharide reaction products formed from Suc at $t = 0$, 1, 3 and 6 h are depicted in Figure 2. Evaluation of these profiles clearly indicated that the α1 ↔ 2β linkage of Suc as donor was split, yielding fructose (Fru) and Glc. Fru accumulated during the reaction; however, Glc remained low, because it was used mainly for the transfer to Suc and its formed gluco-elongations as acceptors, yielding an ensemble of nonreducing gluco-oligosaccharides of increasing DP (as determined by MALDI-TOF-MS analysis) with Fru at the end. Complementary analysis performed by size-exclusion chromatography revealed the presence of two major populations of products, increasing simultaneously during the reaction. A peak with polymeric material eluted from 20 to 24 min and a peak corresponding to oligosaccharides eluted from 30 to 32 min. The polymer formed after 15 min of incubation was estimated to be already larger than 2×10^7 Da (data not shown).

To get insight into the structures of the formed oligosaccharides in the reaction mixtures, the HPAEC fractions 2–11 were isolated and studied by MALDI-TOF-MS and NMR spectroscopy (^{1}H, TOCSY, ^{1}H–^{13}C HSQC and ROESY). In the assignment of the various NMR signals, our earlier developed structural-reporter-group concept for α-D-glucans played an important role (Van Leeuwen, Kralj et al. 2008; Van Leeuwen, Leeflang et al. 2008). Analysis of the peaks after a 1-h incubation showed that Glc is used to elongate Suc with an (α1 → 4)- (Compound 3) or an (α1 → 6)- (Compound 2) linked Glc unit. Later in time ($t = 3$ h, 6 h), more products are formed by elongation of Suc-containing oligosaccharides, whereby the systematic build-up of the structures is impressive. Successive (1 → 4) elongation could be observed only up to DP4 (Compounds 3 and 5), but otherwise the products contained alternating (1 → 6)/(1 → 4) linkages (Compounds 2 → 4 → 5′ → 6 → 7 → 8 → 9 → 10 → 11). Due to the decrease in PAD response with increasing DP of products, together with their low abundance, it was not possible to detect the formation of higher-molecular-mass material by HPAEC. The minor amounts of two non-Fru-containing compounds (the tri- and tetrasaccharide marked with an asterisk in Figure 2, $t = 6$ h) most likely are hydrolysis products of initially formed larger oligosaccharides. It should be noted that Suc was not chemically hydrolyzed in

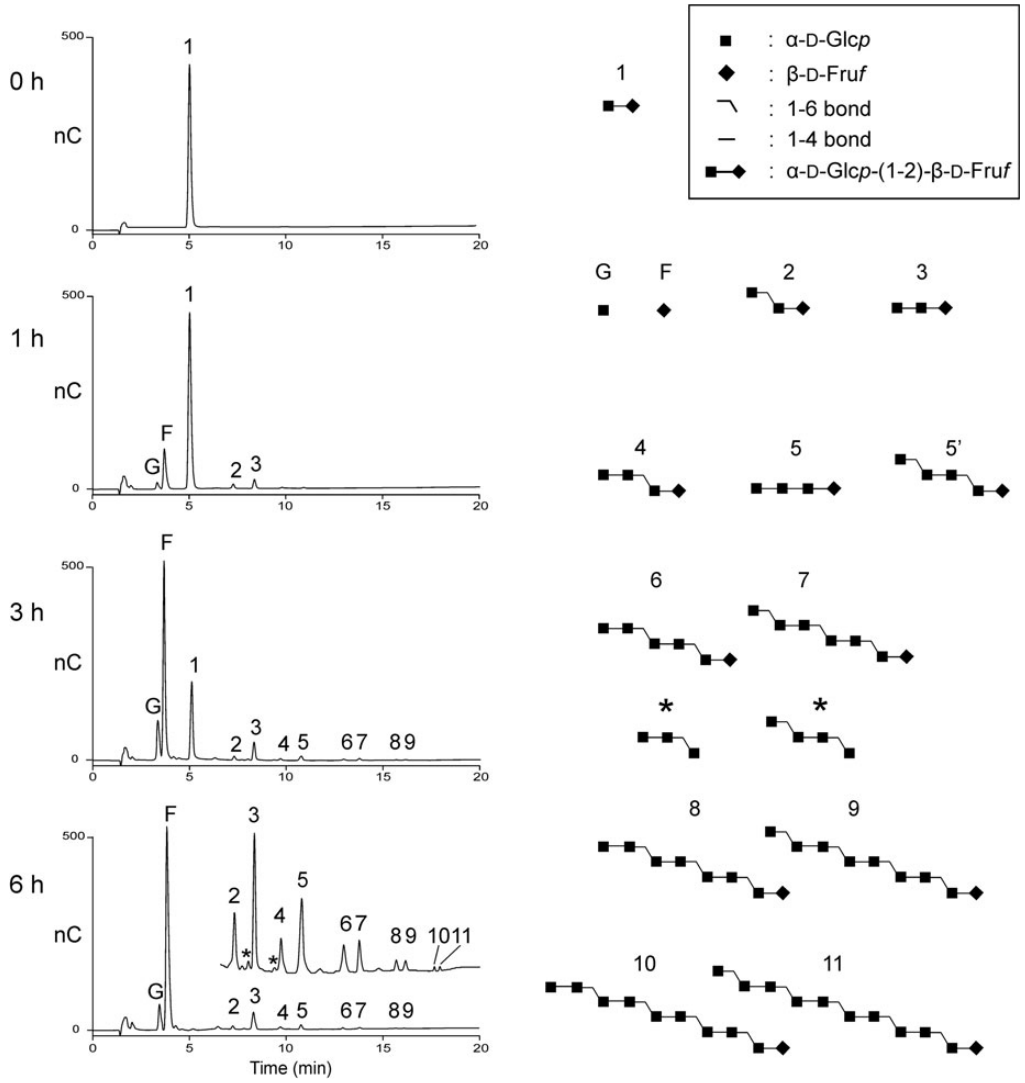

Fig. 2. HPAEC-PAD profile (0–500 mM NaOAc gradient in 100 mM NaOH) on CarboPac PA-1 of the oligosaccharide mixture generated from Suc by incubation with the GTFA enzyme at $t = 0$, 1, 3 and 6 h (pH 4.7, 37°C). Established oligosaccharide structures for isolated fractions are included. G = glucose; F = fructose. Two non-Fru-containing compounds are marked with an asterisk.

the incubation buffer after 6 h at 37°C. Also, enzymatic cleavage of the glycosidic bond in the Suc fragment of higher oligosaccharides is highly improbable. Therefore, the presence of these products indicates that GTFA has a minor endo-(α1 → 4)-glycosidase activity, although it cannot be completely excluded that Glc units are transferred for a very low percentage onto free Glc as an acceptor. Finally, only a minor amount of leucrose (α-D-Glc*p*-(1 → 5)-D-Fru; <2%) was found, indicating that Fru is not a good acceptor substrate for GTFA.

The 1D ^{1}H NMR spectra of Compounds 1–11, including some exact chemical shift values, are depicted in Figure 3. For further NMR data, see Table I. An adequate denotation system for the constituent residues was necessary (see Scheme I), since there are small variations of some of the chemical shift values of the different Glc residues, depending on their position in the oligosaccharide structure.

As an example of the rationalization behind the interpretation of the various ^{1}H and ^{13}C NMR assignments, the TOCSY, ROESY and HSQC spectra of one of the reaction products, Glc_5Suc (Compound 7; *m/z* 1175.3, $[M + Na]^+$ according to MALDI-TOF-MS), are shown in Figure 4. Typical chemical shift values for the Suc fragment (Glc, ***g***; Fru, ***f***) are δ 5.427, stemming from Glc ***g*** H-1 and δ 4.220, stemming from Fru ***f*** H-3. The ^{1}H chemical shifts of the anomeric signals around δ 5.37 and 4.95 indicate the presence of (α1 → 4) and (α1 → 6) linkages, respectively, between Glc residues (Van Leeuwen, Leeflang et al. 2008). Starting from the anomeric signals of the Glc residues $\mathbf{A^X}$, $\mathbf{A^I}$, $\mathbf{B^I}$, $\mathbf{B^{II}}$ and **E** in the 2D ^{1}H–^{1}H TOCSY spectra (20, 50, 100 and 200 ms), and additional data from the HSQC and ROESY experiments, all chemical shifts of the non-anomeric protons of the differently substituted Glc residues could be determined (Table I). Although the anomeric signals

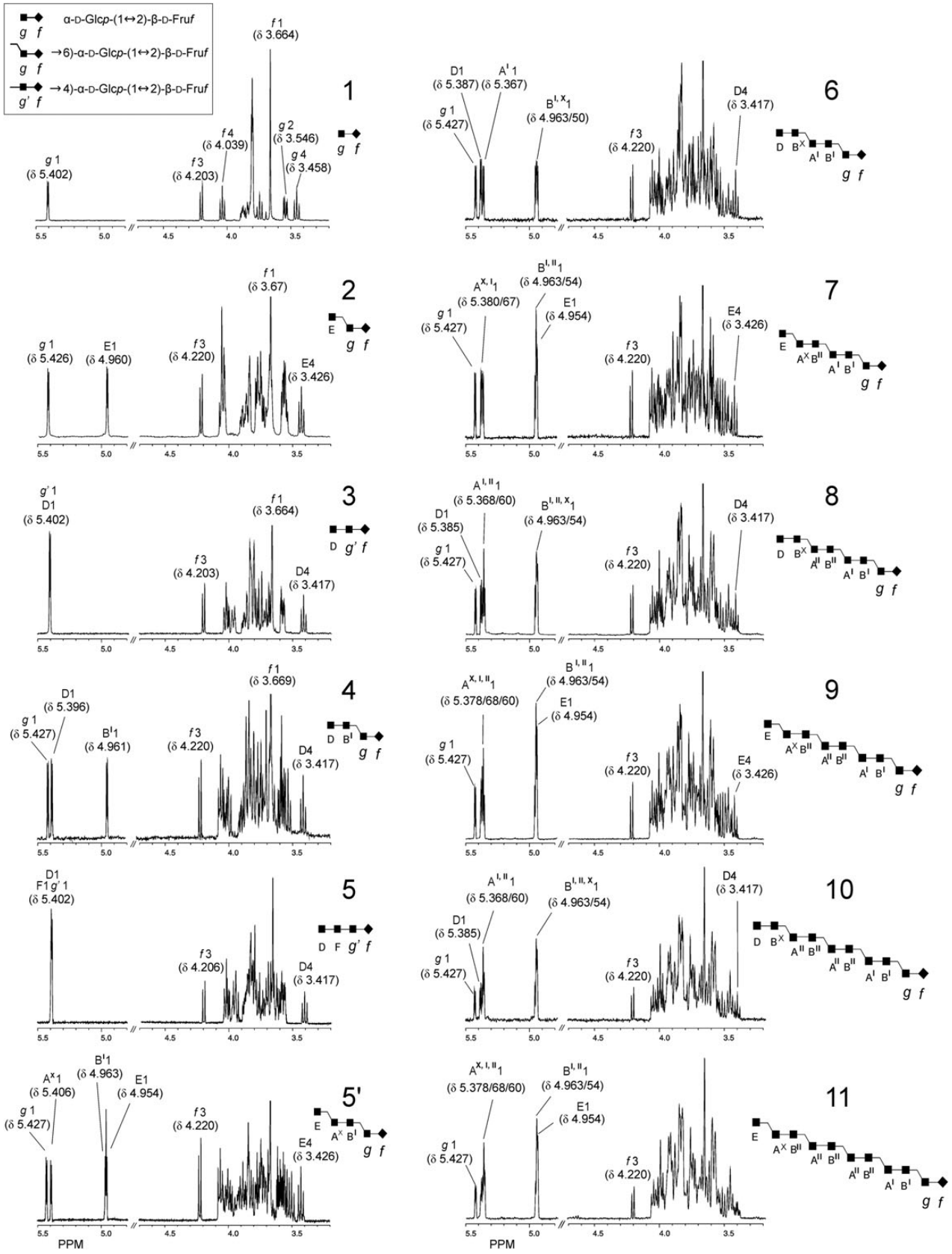

Fig. 3. ^{1}H NMR spectra (D_2O, 300 K) of Compounds 1–11 (Figure 2) found in the oligosaccharide mixture after incubation of Suc with the GTFA enzyme for 6 h (pH 4.7, 37°C).

Table I. ^{1}H and ^{13}C chemical shifts[a] (D_2O, 300 K) of saccharide residues present in linear oligomers (DP ≥ 4) formed by incubation of Suc with the GTFA enzyme of *L. reuteri* 121

Residue	H-1a C-1	H-1b	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6a C-6	H-6b
g	5.427	–	3.57	3.74	3.52	4.05	3.69	4.02
-(1 → 6)-α-D-Glc*p*-	92.9		71.8	73.4	70.5	72.3	66.9	
(1 ↔ 2)-β-D-Fru*f*-								
g′	5.402	–	3.58	4.02	3.69	3.96	3.85	3.76
-(1 → 4)-α-D-Glc*p*-	93.1		72.2	75.1	78.0	72.1	61.5	
(1 ↔ 2)-β-D-Fru*f*-								
f **[*g* → *f*]**	3.67	3.67	-	4.220	4.05	3.90	3.85	3.78
-(1 ↔ 2)-β-D-Fru*f*-	62.5		104.5	77.3	74.9	82.3	63.5	
f **[*g′* → *f*]**	3.66	3.66	-	4.20	4.02	3.89	3.86	3.80
-(1 ↔ 2)-β-D-Fru*f*-	62.5		104.5	77.3	74.9	82.3	63.5	
$\mathbf{A}^{\mathbf{X,I,II}}$	$5.38^{X}/37^{I}/36^{IIb}$	–	3.59	3.69	$3.49^{X}/46^{I,II}$	3.94	$3.73^{X}/77^{I,II}$	$3.98^{X}/94^{I,II}$
-(1 → 6)-α-D-Glc*p*-(1 → 4)-	100.7		72.4	74.3	70.5	72.4	66.5	
$\mathbf{B}^{\mathbf{I,II,X}}$	$4.96^{I}/95^{II,Xb}$	–	3.60	4.01	$3.63^{I}/65^{II,X}$	3.85	3.87	3.83
-(1 → 4)-α-D-Glc*p*-(1 → 6)-	98.9		72.5	74.4	78.5	71.3	61.5	
D	5.40/39[b]	–	3.59	3.67	3.417	3.73	3.84	3.75
α-D-Glc*p*-(1 → 4)-	100.7		72.7	73.8	70.3	73.7	61.5	
E	4.954	–	3.56	3.74	3.426	3.75	3.85	3.77
α-D-Glc*p*-(1 → 6)-	98.8		72.4	72.8	70.5	72.3	61.5	

[a]In ppm relative to the signal of internal acetone (δ 2.225 for ^{1}H and δ 31.07 for ^{13}C).
[b]Average chemical shifts values of the anomeric protons (see exact values: ^{1}H NMR spectra, Figure 3).

Scheme I. Monosaccharide coding system for the discussed oligosaccharides

Residue	Position in the oligosaccharide sequence
g-f	Suc of which Glc (***g***) is substituted at O-6
g′-f	Suc of which Glc (***g′***) is substituted at O-4
$\mathbf{A}^{\mathbf{I}}$	-(1 → 6)-α-D-Glc*p*-(1 → 4)- **A** residue, most close to Suc
$\mathbf{A}^{\mathbf{II,III}}$	More remote -(1 → 6)-α-D-Glc*p*-(1 → 4)- **A** residues
$\mathbf{A}^{\mathbf{X}}$	Sub-terminal -(1 → 6)-α-D-Glc*p*-(1 → 4)- **A** residue
$\mathbf{B}^{\mathbf{I}}$	-(1 → 4)-α-D-Glc*p*-(1 → 6)- **B** residue, most close to Suc
$\mathbf{B}^{\mathbf{II,III}}$	More remote -(1 → 4)-α-D-Glc*p*-(1 → 6)- **B** residues
$\mathbf{B}^{\mathbf{X}}$	Sub-terminal -(1 → 6)-α-D-Glc*p*-(1 → 4)- **B** residue
D	Terminal α-D-Glc*p*-(1→4)- residue
E	Terminal α-D-Glc*p*-(1→6)- residue

of **E** (H-1, δ 4.954) and $\mathbf{B}^{\mathbf{I}}/\mathbf{B}^{\mathbf{II}}$ (H-1, δ 4.963/4.954) strongly overlap, the differences in chemical shift of their H-3, H-4, H-5, H-6 signals could be deduced from the TOCSY built-up series of mixing times (20, 50, 100 and 200 ms; Figure 4, 200 ms). The set of chemical shifts of **E** H-2, H-3, H-4, H-5 and H-6a/b at δ 3.56, 3.74, 3.426, 3.75 and 3.85/3.77, respectively, corresponds with that of a terminal α-D-Glc*p*-(1 → 6)-unit. The set of chemical shifts of $\mathbf{B}^{\mathbf{I}}/\mathbf{B}^{\mathbf{II}}$ H-2, H-3, $H\text{-}4^{I}/H\text{-}4^{II}$, H-5 and H6a/b at δ 3.60, 4.01, $3.63^{I}/3.65^{II}$, 3.85 and 3.87/3.83, respectively, corresponds with that of internal -(1 → 4)-α-D-Glc*p*-(1 → 6)- units. The small differences in chemical shift between $\mathbf{B}^{\mathbf{I}}$ H-1 and $\mathbf{B}^{\mathbf{II}}$ H-1 and between $\mathbf{B}^{\mathbf{I}}$ H-4 and $\mathbf{B}^{\mathbf{II}}$ H-4 are due to the proximity of $\mathbf{B}^{\mathbf{I}}$ to the Suc fragment (ROESY data, see below). The sets of chemical shifts of $\mathbf{A}^{\mathbf{X}}$ and $\mathbf{A}^{\mathbf{I}}$ reflect the presence of internal -(1 → 6)-α-D-Glc*p*-(1 → 4)-units ($\mathbf{A}^{\mathbf{X}}/\mathbf{A}^{\mathbf{I}}$: H-1, δ 5.380/5.367; H-2, δ 3.59; H-3, δ 3.69; $H\text{-}4^{X}/H\text{-}4^{I}$, δ 3.49/3.46; H-5, δ 3.94; $H6a^{X}/b^{X}$ and $H6a^{I}/b^{I}$, δ $3.73^{X}/3.98^{X}$ and δ $3.77^{I}/3.94^{I}$). In this case, the small differences in chemical shift for H-1, H-4 and H6a/b are due to the proximity of $\mathbf{A}^{\mathbf{I}}$ to the Suc fragment (ROESY data, see below).

The set of chemical shifts of ***g*** H-2, H-3, H-4, H-5 and H-6a/b at δ 3.57, 3.74, 3.52, 4.05 and 3.69/4.02, respectively, corresponds with a 6-*O*-substituted ***g*** residue [-(1 → 6)-α-D-Glc*p*-(1 ↔ 2)-β-D-Fru*f*]. The TOCSY ***f*** H-3 track (δ 4.220) revealed cross-peaks with ***f*** H-4, ***f*** H-5 and ***f*** H-6a/b at δ 4.05, 3.90 and 3.85/3.78, respectively, in agreement with literature data (Timmermans et al. 1993).

The sequence of the five Glc residues followed directly from the ROESY experiments, revealing inter-residual cross-peaks between **E** H-1 and $\mathbf{A}^{\mathbf{X}}$ H-6a ($\mathbf{E}1 \rightarrow 6\mathbf{A}^{\mathbf{X}}$), $\mathbf{A}^{\mathbf{X}}$ H-1 and $\mathbf{B}^{\mathbf{II}}$ H-4 ($\mathbf{A}^{\mathbf{X}}1 \rightarrow 4\mathbf{B}^{\mathbf{II}}$), $\mathbf{B}^{\mathbf{II}}$ H-1 and $\mathbf{A}^{\mathbf{I}}$ H-6a ($\mathbf{B}^{\mathbf{II}}1 \rightarrow 6\mathbf{A}^{\mathbf{I}}$), $\mathbf{A}^{\mathbf{I}}$ H-1 and $\mathbf{B}^{\mathbf{I}}$ H-4 ($\mathbf{A}^{\mathbf{I}}1 \rightarrow 4\mathbf{B}^{\mathbf{I}}$) and $\mathbf{B}^{\mathbf{I}}$ H-1 and ***g*** H-6a ($\mathbf{B}^{\mathbf{I}}1 \rightarrow 6\boldsymbol{g}$). The 4- and 6-substitutions of the residues $\mathbf{B}^{\mathbf{I}}/\mathbf{B}^{\mathbf{II}}$ and $\mathbf{A}^{\mathbf{X}}/\mathbf{A}^{\mathbf{I}}/\boldsymbol{g}$, respectively, were further supported by their downfield C-4 and C-6 chemical shifts, compared with terminal Glc (deduced from ^{13}C–^{1}H HSQC measurements): $\mathbf{B}^{\mathbf{I}}/\mathbf{B}^{\mathbf{II}}$ C-4 at δ 78.5 and $\mathbf{A}^{\mathbf{X}}/\mathbf{A}^{\mathbf{I}}/\boldsymbol{g}$ C-6 at δ 66.5/66.9.

In conclusion, the NMR chemical shift data, together with the peak areas of the anomeric signals and the molecular mass (1152 Da), revealed the complete structure of the oligosaccharide, being a linear heptasaccharide consisting of Suc elongated by five Glc residues with alternating (α1 → 6)/(α1 → 4) glycosidic linkages: α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 ↔ 2)-β-D-Fru*f*.

The data show that during the first 6 h of incubation with Suc, GTFA mainly produces linear oligosaccharides with alternating (1 → 6)/(1 → 4) linkages; no branched oligosaccharides were formed. However, the consecutively formed polymeric material starting from DP14 on clearly contained → 4,6)-branched Glc residues, as indicated by ^{1}H-NMR signals at δ 5.33. The degree of branching may increase during the further growth of the glucan chain. Up to DP20, the presence of the Suc fragment in the structures could be observed in the NMR

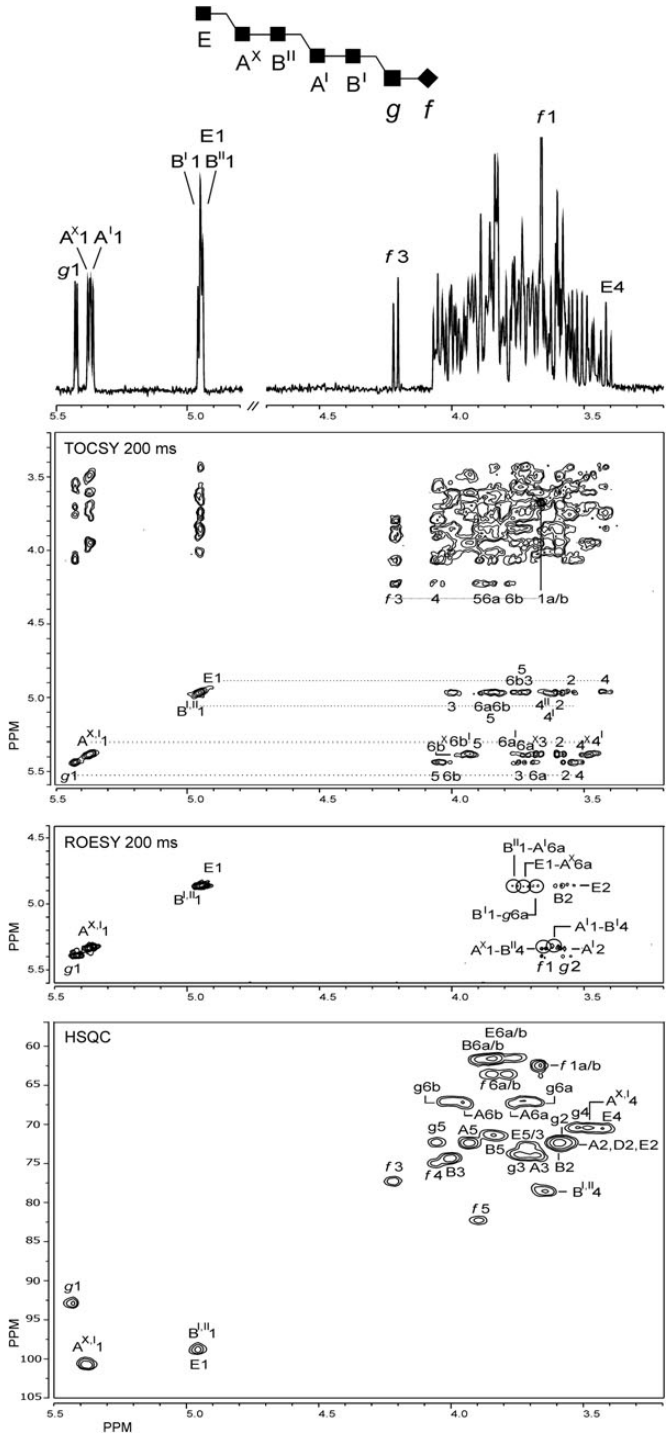

Fig. 4. 1D ^{1}H NMR, TOCSY (200 ms), ROESY (200 ms) and HSQC spectra of α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 4)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 ↔ 2)-β-D-Fru*f* (Compound 7 in Figure 2).

spectra by the small signal at δ 4.220 stemming from Fru*f* H-3. The polysaccharide material at 72 h of incubation was isolated via size-exclusion chromatography on Bio-Gel P2 by collecting the void-volume fraction. The 1D ^{1}H NMR spectrum (Figure 5) of this fraction was identical to the spectrum of reuteran,

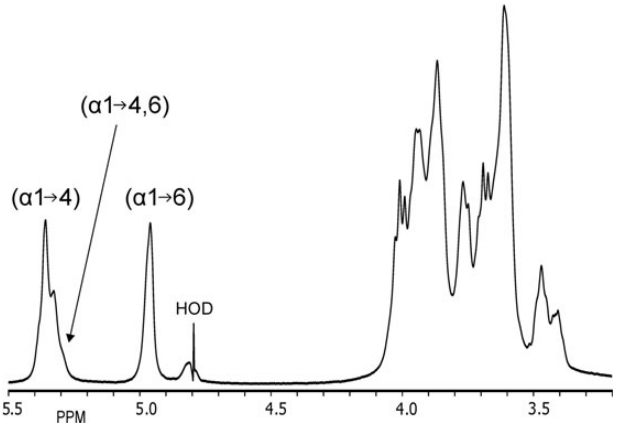

Fig. 5. ^{1}H NMR spectrum of the void-volume Bio-Gel P-2 polysaccharide fraction, obtained after 72 h incubation of Suc with the GTFA enzyme. The spectrum is identical to the ^{1}H NMR spectrum of the native polysaccharide produced by *L. reuteri* 121 (Van Leeuwen, Kralj et al. 2008).

containing 14% of →4,6)-branching, for which the composite model is depicted in Figure 1 (Van Leeuwen, Kralj et al. 2008). Note that the NMR signals of Suc/Fru are no longer observable in the NMR spectrum of the polysaccharide.

Products of the GTFA enzyme from malto-oligosaccharide substrates in the absence of Suc

To generate more information about the earlier observed activity of recombinant GTFA with MOSs, a detailed structural analysis of products formed was carried out. To this end, 100 mM solutions of MOS (DP2–DP6) were individually incubated for different times up to 72 h at 37°C with 50 nM recombinant GTFA in 25 mM sodium acetate buffer/1 mM $CaCl_2$, pH 4.7. TLC analysis (data not shown) of the various incubation mixtures revealed that only for MOS DP > 3, the GTFA enzyme clearly possessed hydrolysis/transferase activity. GTFA converted only minor amounts of maltose and maltotriose. For MOS DP4–DP6, the yield of product formation was higher but still less than observed during the incubation of Suc together with maltose (see below) under the same conditions and incubation time. GTFA thus has relatively minor activity with these MOS substrates.

Incubation of maltose (MOS DP2) with the GTFA enzyme for 24 h on a semi-preparative scale, followed by HPAEC profiling, product isolation, MALDI-TOF-MS and NMR analysis, showed only minimal formation of Glc and panose [α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 4)-D-Glc*p*] (Compound 3) (Figure 6), indicating that GTFA is poorly hydrolyzing maltose. HPAEC-PAD profiling of the incubations of GTFA with MOS DP3–DP6 over periods up to 30 h gave more insight into the type of products formed. The chromatograms of the incubations of DP3 and DP4 at relevant incubation times are shown in Supplementary data, Figure S1 and Figure 7, respectively. Most of the fractions isolated were analyzed by MALDI-TOF-MS and 1D/2D NMR spectroscopy (including TOCSY, ^{1}H–^{13}C HSQC and ROESY experiments). For general ^{1}H and ^{13}C NMR data, see Tables II and III. The established structures are included in the various figures.

Next to the hydrolysis products Glc (Compound 2) and maltose (Compound 3), the transfer product (Compound 4) formed from maltotriose (MOS DP3) at t = 6 h is maltotetraose

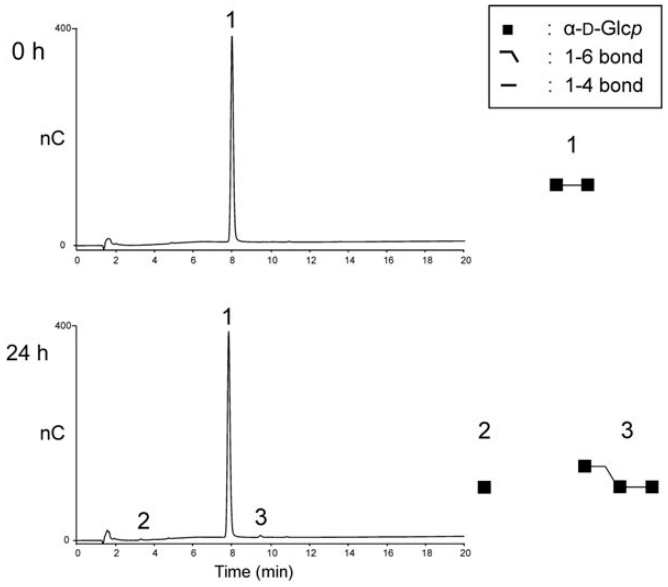

Fig. 6. HPAEC-PAD profile (0–500 mM NaOAc gradient in 100 mM NaOH) on CarboPac PA-1 of the oligosaccharide mixture generated from maltose (MOS DP2) with the GTFA enzyme at $t = 0$ and 24 h (pH 4.7, 37°C). Established oligosaccharide structures for isolated fractions are included.

(MOS DP4), the result of an (α1 → 4) → (α1 → 4) transfer of a single α-D-Glc*p* unit (Supplementary data, Figure S1). After 24 h, an (α1 → 4) → (α1 → 6) linkage transfer of a single α-D-Glc*p* unit to the nonreducing end and an (α1 → 4) → (α1 → 2) linkage transfer of a single α-D-Glc*p* unit to the reducing end of maltotriose was observed. Furthermore, panose (Compound 5) was detected, showing that formed maltose also acts as an acceptor for an (α1 → 6) elongation, in agreement with the direct incubation of maltose with GTFA.

Incubation of maltotetraose (MOS DP4) with GTFA yielded, after 30 h, an interesting ensemble of products (MALDI-TOF-MS showed DP2–DP6; $[M + Na]^+$, *m/z* 365.2–1661.1); the major higher-molecular-mass product at $t = 12$ h comprised an (α1 → 4) → (α1 → 6) linkage transfer of a single α-D-Glc*p* unit to the nonreducing end of maltotetraose (Compound 4 in Figure 7). As can be seen from the sixteen isolated products characterized (Figure 7), GTFA displays hydrolysis (Compounds 2, 3 and 9), as well as disproportionation activity, namely elongations with α-D-Glc*p*-(1 → 6) (Compounds 4, 6, 7, 10 and 11) and α-D-Glc*p*-(1 → 4) (Compound 5) units at the nonreducing end, as well as elongations with a single α-D-Glc*p*-(1 → 2) unit at the reducing end (Compounds 8, 12 and 13). After prolonged incubation, small amounts of higher-molecular-mass isomalto-/malto-oligomers were detected (Compounds 14, 15 and 16), a type of structure that has been found earlier as products from incubations of MOS with GTFB, although with longer sequences of (1 → 6)-linked α-D-Glc*p* units (Dobruchowska et al. 2012). GTFA, thus, also has (minor) 4,6-α-glucanotransferase activity. Note that after 30 h, the original donor substrate maltotetraose (Compound 1) had nearly disappeared. Higher polymeric material could not be detected: Only oligosaccharide products (up to DP10) were observed. The 1D ^{1}H NMR spectra of Compounds 4 and 16 (Figure 7), including some exact chemical shift values, are depicted in Supplementary data, Figure S2.

To illustrate the structure determination of oligosaccharides with a (1 → 2)-linked α-D-Glc*p* residue in a reducing end → 2,4)-D-Glc*p* situation, the NMR analysis of Compound 13 (Figure 7) (tetrasaccharide; MALDI-TOF-MS *m/z* 689.3, $[M + Na]^+$) is presented here in more detail. The 1D ^{1}H NMR spectrum of Compound 13, together with the TOCSY (200 ms), ROESY (200 ms) and HSQC spectra, is depicted in Figure 8, and the various ^{1}H and ^{13}C NMR data are presented in Table II.

The 1D ^{1}H NMR spectrum of Compound 13 showed 6 anomeric signals, which were used as starting points for the assignment of the chemical shifts of all nonanomeric protons in the 2D TOCSY spectrum (TOCSY spectra with spin-lock times 20, 50, 100 and 200 ms were used to acquire all assignments). The H-1 signals at δ 5.441 (**R**α1) and 4.809 (**R**β1) are in agreement with a reducing -(1 → 2,4)-D-Glc*p* **R** unit (http://www.casper.organ.su.se/casper/; Jansson et al. 2006). The splitting of the **G** H-1 doublet into two doublets ($\mathbf{G}_\alpha$ H-1, δ 5.099 and $\mathbf{G}_\beta$ H-1, δ 5.387) corresponds with the long-range effect induced by the α- and β-anomeric forms of residue **R** (Van Leeuwen, Leeflang et al. 2008). The anomeric signals at δ 5.433 and 4.958 reflect the presence of an internal -(1 → 6)-α-D-Glc*p*-(1 → 4)- unit and a terminal α-D-Glc*p*-(1 → 6)- unit, respectively (Dobruchowska et al. 2012). In the 2D ROESY spectrum, strong inter-residual couplings were observed between $\mathbf{G}_\alpha$ H-1 and **R**α H-2, $\mathbf{G}_\beta$ H-1 and **R**β H-2, **A** H-1 and **R**α H-4, **A** H-1 and **R**β H-4 and **E** H-1 and **A** H-6a/b, in accordance with the structure α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 4)-[α-D-Glc*p*-(1 → 2)-]D-Glc*p*. The various substitution patterns are confirmed by their ^{13}C NMR data, deduced from the ^{13}C–^{1}H HSQC spectrum: **R**α (C-2, δ 76.5; C-4, δ 77.5), **R**β (C-2, δ 79.5; C-4, δ 77.5), **A** (C-6, δ 66.7) (Bock and Thøgersen 1982; Van Leeuwen, Leeflang et al. 2008; Dobruchowska et al. 2012).

Incubation of maltohexaose (MOS DP6) with GTFA yielded, after 30 h, 23 isolated products (Figure 9) (MALDI-TOF-MS: DP2–DP9; $[M + Na]^+$, *m/z* 365.3–1661.2), which were subjected to a detailed structural analysis. The major compounds comprised structures 2 (Glc), 8 (maltose), 7 (panose), 6 (maltotriose), 3 (maltopentaose), 5 (maltopentaose elongated with an (1 → 6)-linked α-D-Glc*p* unit), 1 (maltohexaose) and 4 (maltohexaose elongated with an (1 → 6)-linked α-D-Glc*p* unit), indicating hydrolysis activity and an (α1 → 4) → (α1 → 6) linkage transfer of a single α-D-Glc*p* unit. Besides structures similar to those seen for MOS DP4, including relatively minor products with an (1 → 2)-linked α-D-Glc*p* unit (Compounds 10, 11 and 13), a minor 4,6-branched structure was identified (Compound 22). Interestingly, in this case also minor structures with alternating (α1 → 6)/(α1 → 4) linkages were detected (for NMR details, see Table III). Linear sequences of (1 → 6)-linked α-D-Glc*p* units, as seen in the incubation of MOS DP4 with GTFA, were not observed with MOS DP6. 1D ^{1}H NMR spectra of Compounds 19, 21, 22 and 23 (Figure 9), including some exact chemical shift values, are depicted in Supplementary data, Figure S2.

Products of the GTFA enzyme incubated with MOSs plus Suc as substrates

Previously, it has been observed that the presence of both maltose and Suc influences the GTFA-catalyzed spectrum of generated oligosaccharides, compared with Suc alone as

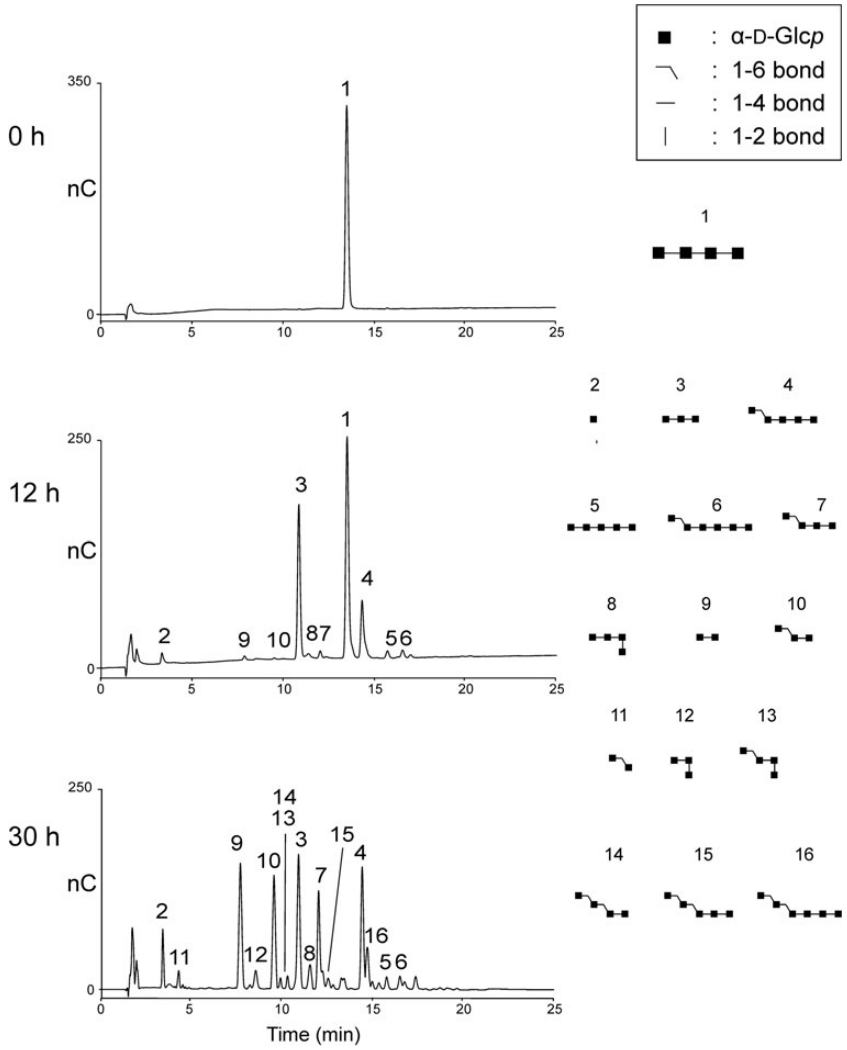

Fig. 7. HPAEC-PAD profile (0–500 mM NaOAc gradient in 100 mM NaOH) on CarboPac PA-1 of the oligosaccharide mixture generated from maltotetraose (MOS DP4) with the GTFA enzyme at $t = 0$, 12 and 30 h (pH 4.7, 37°C). Established oligosaccharide structures for isolated fractions are included.

substrate (Kralj, van Geel-Schutten, van der Maarel et al. 2004). For a more detailed structural analysis, solutions of 100 mM sucrose and 100 mM MOS (DP2–DP6) were each incubated with 50 nM recombinant GTFA in 25 mM sodium acetate buffer/1 mM $CaCl_2$, pH 4.7, at 37°C. The reaction was followed in time by TLC screening (data not shown).

Inspection of the HPAEC profiles of products formed after 1, 3 and 6 h incubation of sucrose and maltose with GTFA (Figure 10) showed the formation of a series of products up to DP7, and after 6 h, the complete disappearance of Suc. Prolonged incubation resulted in an increase in molecular size of the products, but the amounts were very low (according to TLC and MALDI-TOF-MS screening), and these were not studied in more detail. Most of the products formed within 6 h were isolated and investigated by MALDI-TOF-MS and 1D/2D NMR spectroscopy. Surprisingly, the majority of the initially formed compounds did not stem from elongations of Suc, but from extensions of maltose by Glc residues. Apparently, GTFA cleaves Suc and the Glc units generated are preferentially used to elongate maltose instead of Suc. Only traces of Suc elongations, i.e., two trisaccharides (marked with an asterisk, Figure 10) were detected. Fru and panose (Compound 3 in Figure 10) were found as the most abundant products. The (α1 → 4)/(α1 → 6) alternating build-up of the structures of the minor oligosaccharides 5, 6, 11 and 12 in Figure 10 is similar to the Suc-containing structures found for the Suc incubation with GTFA (Figure 2). In contrast to the "sucrose-only" incubation, just a minor amount of polymeric material (DP > 20 according to MALDI-TOF-MS) was detected after prolonged incubations, and now a → 4,6)-branched structure was already seen after 6 h of incubation (structure 5′, Figure 10). Also in this case, the three structures with (1 → 2)-linked α-D-Glc*p*, earlier found in the GTFA incubations with maltotriose (only one of the three), maltotetraose and maltohexaose (Supplementary data, Figure S1; Figures 7 and 9), were detected. Compound 9 (Figure 10) was also traced in the incubation of maltotetraose with GTFA (Compound 14 in Figure 7), showing the possibilities of synthesizing linear sequences of (1 → 6)-linked α-D-Glc*p* units. For a survey of the

Table II. ^{1}H and ^{13}C chemical shifts[a] (D_2O, 310 K) of Glc residues present in -2,4)-substituted-oligomers formed by incubation of MOSs (with and without Suc) with the GTFA enzyme of *L. reuteri* 121

Residue	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6a C-6	H-6b
A	5.433	3.58	3.68	3.50	3.90	3.74	3.98
-(1 → 6)-α-D-Glc*p*-(1 → 4)-	100.5	72.4	73.3	70.2	71.1	66.7	
D	5.45/40[b]	3.59	3.69	3.43	3.73	3.85	3.77
α-D-Glc*p*-(1 → 4)-	100.6	72.5	73.8	70.2	73.6	61.3	
E	4.958	3.56	3.74	3.43	3.72	3.85	3.76
α-D-Glc*p*-(1 → 6)-	99.0	72.3	73.9	70.3	72.6	61.2	
F	5.432	3.61	3.96	3.65	3.85	3.89	3.82
-(1 → 4)-α-D-Glc*p*-(1 → 4)-	100.0	72.3	74.1	77.7	72.0	61.3	
Rα	5.44[b]	3.68	4.09	3.71	3.97	3.87	3.83
-(1 → 2,4)-α-D-Glc*p*	90.1	76.5	72.5	77.5	70.6	61.2	
Rβ	4.809	3.43	3.87	3.68	3.60	3.94	3.77
-(1 → 2,4)-β-D-Glc*p*	97.0	79.5	75.8	77.5	75.3	61.7	
$G_α$	5.099	3.56	3.78	3.45	3.91	3.85	3.80
α-D-Glc*p*-(1 → 2)-	97.2	72.3	73.6	70.3	72.7	61.2	
$G_β$	5.40/38[b]	3.55	3.75	3.45	3.99	3.82	3.79
α-D-Glc*p*-(1 → 2)-	98.6	72.3	73.6	70.3	72.5	61.2	

[a]In ppm relative to the signal of internal acetone (δ 2.225 for ^{1}H and δ 31.07 for ^{13}C).
[b]Average chemical shifts values of the anomeric protons (see exact values: ^{1}H NMR spectra, Supplementary data, Figure S4).

Table III. ^{1}H and ^{13}C chemical shifts[a] (D_2O, 300 K) of Glc residues present in oligomers (DP ≥ 4) formed by incubation of MOS (with and without Suc) with the GTFA enzyme of *L. reuteri* 121

Residue	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6a C-6	H-6b
$A^{I,II,III,X}$	$5.40^{X}/38^{I,III}/36^{IIb}$	3.59	3.69	$3.49^{X}/46^{I,II,III}$	3.94	$3.73^{X}/77^{I,II,III}$	$3.98^{X}/94^{I,II,III}$
-(1 → 6)-α-D-Glc*p*-(1 → 4)-	101.0	72.7	74.1	70.4	72.4	$66.9^{X}/67.3^{I,II,III}$	$66.9^{X}/67.3^{I,II,III}$
$B^{II,X}$	$4.98/95^{II,Xb}$	3.60	4.00	3.65	3.85	3.89	3.82
-(1 → 4)-α-D-Glc*p*-(1 → 6)-	99.0	72.2	74.4	78.5	71.4	61.5	
C	5.36[b]	3.65	3.97	3.74	4.01	3.83	4.03
-(1→4,6)-α-D-Glc*p*-(1→4)-	-	-	-	-	-	-	
D	5.39/38/34[b]	3.59	3.67	3.42	3.74	3.84	3.75
α-D-Glc*p*-(1 → 4)-	99.0	72.5	73.9	70.5	73.9	61.5	
E	4.99/95[b]	3.56	3.75	3.43	3.75	3.85	3.77
α-D-Glc*p*-(1 → 6)-	99.0	72.5	72.7	70.5	72.4	61.5	
F	5.39[b]	3.62	3.96	3.66	3.84	3.88	3.81
-(1 → 4)-α-D-Glc*p*-(1 → 4)-	100.7	72.5	74.3	77.8	72.2	61.5	
J	4.955	3.58	3.70	3.51	3.91	3.76	3.98
-(1→6)-α-D-Glc*p*-(1→6)-	98.8	72.3	74.3	70.6	71.2	66.7	
Rα	5.22/23[b]	3.56	3.97	3.61	3.95	3.88	3.83
-(1 → 4)-α-D-Glc*p*	92.9	72.3	74.2	78.5	71.0	61.5	
Rβ	4.650	3.27	3.77	3.62	3.61	3.94	3.80
-(1 → 4)-β-D-Glc*p*	96.8	75.0	77.2	78.5	75.6	61.9	

[a]In ppm relative to the signal of internal acetone (δ 2.225 for ^{1}H and δ 31.07 for ^{13}C).
[b]Average chemical shifts values of the anomeric protons (see exact values: ^{1}H NMR spectra, Supplementary data, Figures S2 and S3).

^{1}H and ^{13}C NMR data of the various compounds identified, see Table II [→2,4)-branched oligosaccharide products] and Table III [alternating (α1 → 4) and (α1 → 6) linkages]. For a survey of some typical 1D ^{1}H NMR spectra (Compounds 5, 5′, 6, 7, 8, 10, 11 and 12 in Figure 10), including some exact chemical shift values, see Supplementary data, Figures S3 and S4.

Incubation of GTFA with Suc/MOS DP3–DP6 resulted in transferase activity in each case, yielding products similar (data not shown) to those described for the Suc/MOS DP2 experiment, confirming the GTFA preference for Glc transfer to MOS acceptors above transfer to Suc. This clearly reflects a higher affinity of GTFA for MOS than for Suc as acceptor substrate.

Discussion

Detailed characterization of the initially formed oligosaccharides in the biosynthesis of reuteran (EPS121, EPS35-5) from Suc with the *L. reuteri* GTFA enzyme as catalyst clearly shows that it possesses mainly transferase activity. Under the applied conditions, GTFA transfers Glc residues from donor Suc to acceptor Suc and to the growing glucan chain attached to a Suc. Such a reaction was suggested earlier in a study of the mechanism of a *Leuconostoc mesenteroides* NRRL B-512F dextranase and a *L. mesenteroides* NRRL B-1355 alternansucrase, whereby product oligosaccharides of unknown structure were found, of

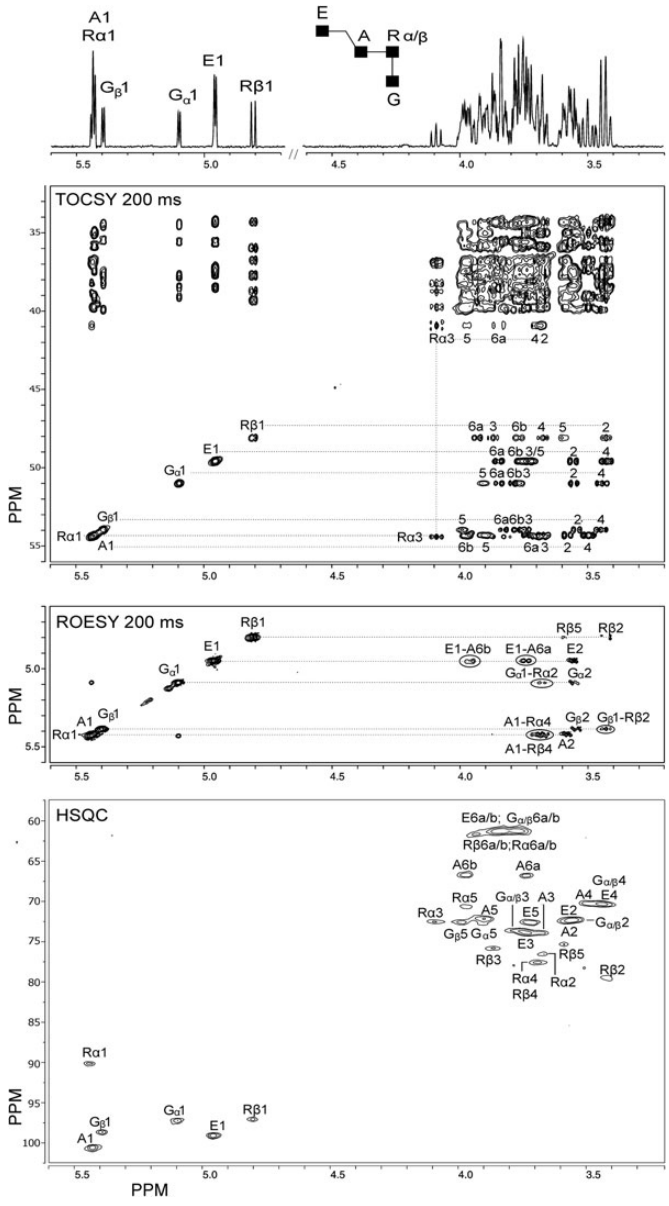

Fig. 8. 1D ^{1}H NMR, TOCSY (200 ms), ROESY (200 ms) and HSQC spectra of α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 4)-[α-D-Glc*p*-(1 → 2)]-α/β-D-Glc*p* (Compound 13 in Figure 7).

which methylation analysis and NMR spectroscopy showed the presence of Fru or Suc located at the oligosaccharide extremity (Moulis et al. 2006).

Incubation of Suc with the GTFA enzyme resulted initially in synthesis of oligosaccharides that were all linear. The transferred Glc residues were mainly attached in alternating (α1 → 6)/(α1 → 4) linkages, forming a Suc-containing oligo-/polysaccharide; no linear (α1 → 6) sequences were found. This observation provides important new insights into the reaction specificity of the GTFA reuteransucrase enzyme. Apparently, the GTFA reaction mechanism seems to dictate alternate binding of the growing linear oligosaccharide acceptor substrates in two different ways in or near the active site, resulting in alternate formation of (α1 → 6) and (α1 → 4) linkages. Our ongoing studies aim to identify these different binding possibilities. After growth of the oligomer up to DP14, →4,6)-branching occurred and increased during further growth of the polymer (DP > 20) to an ultimate 14% branching content. Currently, experiments are in progress to investigate the effects of different Suc concentrations on the synthesis of these oligo/polysaccharides and to obtain further information about the branching reaction occurring during prolonged incubation. Also, the GTFA structural features determining the molecular mass of its EPS formed remain to be elucidated.

Replacement of Suc by MOSs (DP2–DP6) in the incubation with GTFA showed that the latter compounds were relatively poor substrates, and very low amounts of only relatively short oligosaccharides (<DP10) were generated in time; no polymer formation was seen and the major reaction was hydrolysis. The established structures showed besides (α1 → 4) linkages, elongations at the nonreducing end with (1 → 6)-linked α-D-Glc*p* and at the reducing end with (1 → 2)-linked α-D-Glc*p* residues. It should be noted that (α1 → 2) linkages have not been found in the detailed structural analysis of reuteran (EPS121, EPS35-5) (Van Leeuwen, Kralj et al. 2008). So this minor (α1 → 2)-glucosylation activity of GTFA is only seen when it is incubated with relatively high concentrations of MOS, containing a reducing Glc residue. Recently, the 3D structure of an (α1 → 2)-branching glucansucrase, isolated from a *L. mesenteroides* strain, transferring Glc units from Suc onto gluco-oligosaccharides, has been reported (Brison et al. 2012, 2013).

Incubations of GTFA with MOS plus equimolar amounts of Suc revealed that the enzyme initially uses Suc as Glc donor and MOS as acceptor substrates. Previous studies have shown that maltose is the most effective acceptor for an alternansucrase enzyme of a *L. mesenteroides* strain (Côté et al. 2008).

The GTFA enzyme clearly forms novel and interesting (alternan) oligosaccharide mixtures. These compounds will be further evaluated for their prebiotic properties and ability to reduce the blood Glc levels after food consumption (Dijkhuizen et al. 2010; Monsan and Ouarné 2010; Monsan et al. 2010; Leemhuis, Pijning et al. 2013).

Materials and methods

Preparation and isolation of recombinant GTFA enzyme

The recombinant N-terminally truncated GTFA enzyme was prepared and isolated as previously described (Kralj, van Geel-Schutten, Dondorff et al. 2004).

Incubation of Suc and MOSs with GTFA

Before incubation, commercially available MOSs (DP2–DP6) were purified via size-exclusion chromatography on Bio-Gel P-2 (DP2–DP4) or via HPAEC on CarboPac PA-1 (DP5 and DP6). Solutions of Suc (50 mM) and the purified MOSs (100 mM) were individually incubated with 50 nM GTFA in 25 mM sodium acetate/1 mM $CaCl_2$, pH 4.7, at 37°C. The progress of the reactions was followed by analyzing aliquots of the incubation mixtures by TLC, MALDI-TOF-MS and HPAEC-PAD. Furthermore, mixtures of malto-oligosacharides (DP2–DP6, 100 mM), individually together with Suc (100 mM), were incubated in a similar way. Comparable incubations without the addition of GTFA showed no product formation.

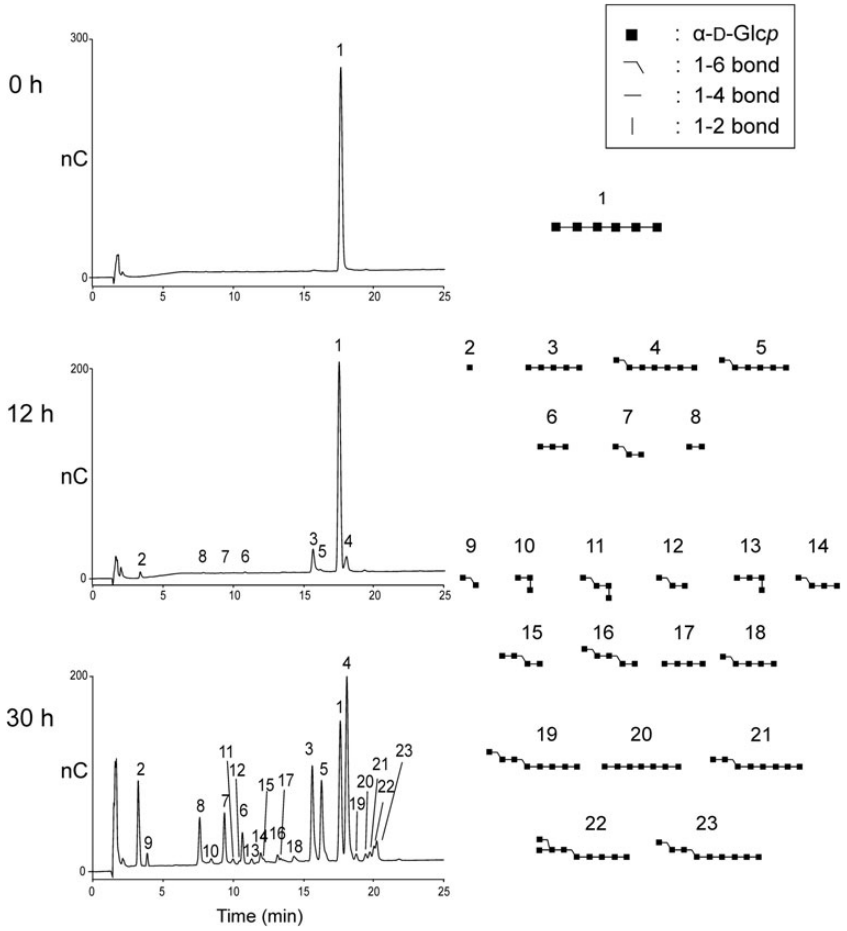

Fig. 9. HPAEC-PAD profile (0–500 mM NaOAc gradient in 100 mM NaOH) on CarboPac PA-1 of the oligosaccharide mixture generated from maltohexaose (MOS DP6) with the GTFA enzyme at $t = 0$, 12 and 30 h (pH 4.7, 37°C). Established oligosaccharide structures for isolated fractions are included.

Isolation and purification of product oligosaccharides

Product mixtures of oligosaccharides obtained after incubation were fractionated by HPAEC on a Dionex DX500 workstation (Dionex, Amsterdam, The Netherlands), equipped with a CarboPac PA-1 column (250 × 9 mm; Dionex) and an ED40 pulsed amperometric detector (PAD), using a linear gradient of 0–500 mM sodium acetate in 100 mM NaOH (3 mL/min) or isocratic conditions of 100 mM sodium acetate in 100 mM NaOH (3 mL/min). Collected fractions were immediately neutralized with 4 M acetic acid, desalted on CarboGraph SPE columns (Alltech, Breda, The Netherlands) using acetonitrile: water = 1:3 as eluent and lyophilized. Polysaccharide material (DP > 14) was isolated in the void volume on a Bio-Gel P-2 column (90 × 1 cm), eluted with 10 mM NH_4HCO_3 at a flow rate of 12 mL/h.

Thin-layer chromatography

Samples were spotted in 1-cm lines on TLC sheets (Merck Kieselgel 60 F254, 20 × 20 cm), which were developed with n-butanol:acetic acid:water = 2:1:1. Bands were visualized by orcinol/sulfuric acid staining and compared with a simultaneous run of standard oligosaccharides.

Matrix-assisted laser-desorption ionization time-of-flight mass spectrometry

MALDI-TOF-MS experiments were performed on an Axima™ mass spectrometer (Shimadzu Kratos, Inc., Manchester, UK) equipped with a nitrogen laser (337 nm, 3 ns pulse width). Positive-ion mode spectra were recorded using the reflector mode at a resolution of 5000 full width at half maximum and delayed extraction (450 ns). The accelerating voltage was 19 kV with a grid voltage of 75.2%; the mirror voltage ratio was 1.12, and the acquisition mass range was 200–6000 Da. Samples were prepared by mixing on the target 0.5 µL sample solutions with 0.5 µL aqueous 10% 2,5-dihydroxybenzoic acid as matrix solution.

NMR spectroscopy

Resolution-enhanced 1D/2D 500-MHz ^{1}H NMR spectra were recorded in D_2O on a Bruker DRX-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University) or a Varian Inova Spectrometer (NMR Center, University of Groningen) at probe temperatures of 300/310 K. Before analysis, samples were exchanged twice in D_2O (99.9 atom% D, Cambridge Isotope Laboratories, Inc., Andover, MA) with intermediate lyophilization, and then dissolved in 0.6 mL

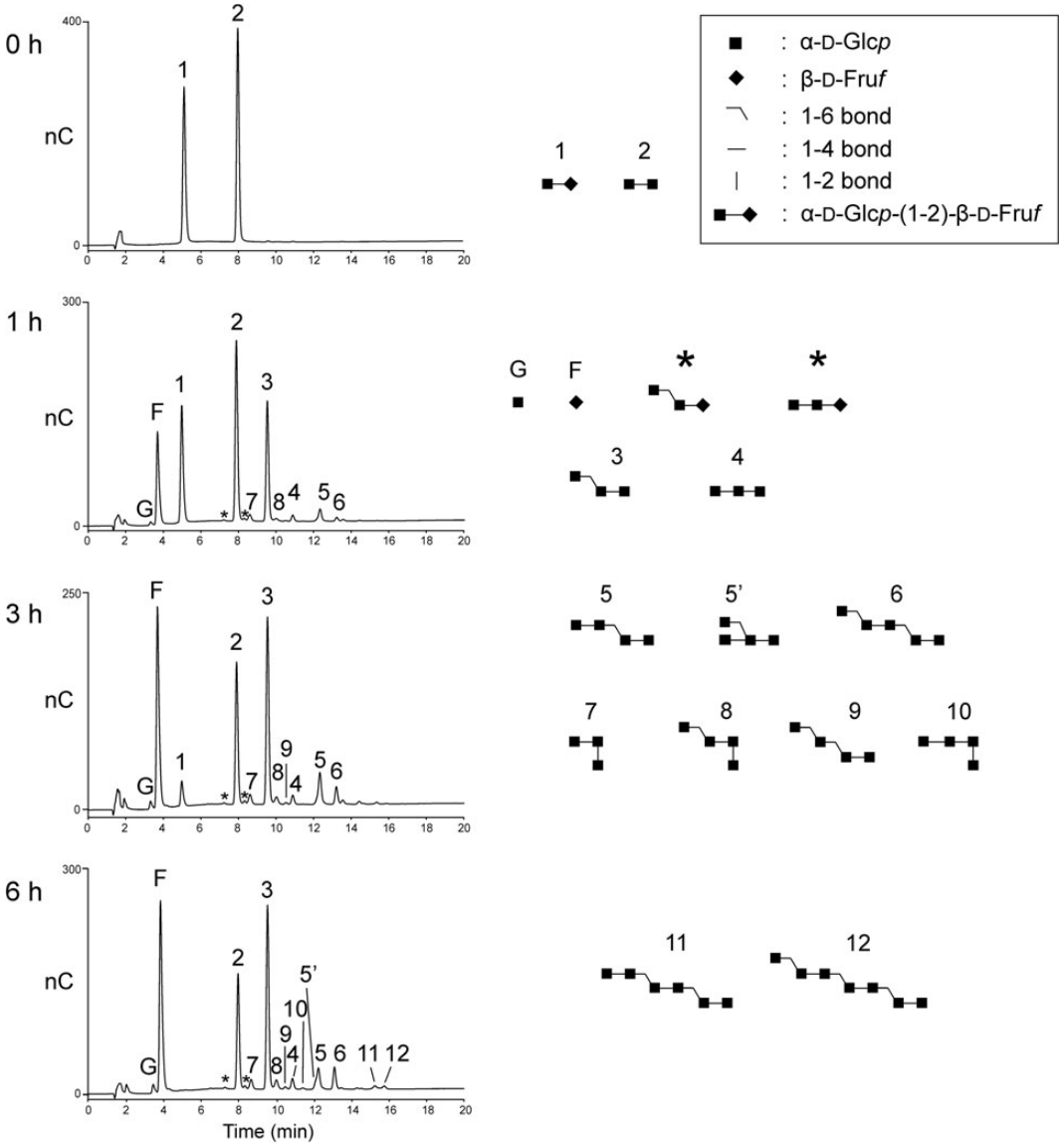

Fig. 10. HPAEC-PAD profile (0–500 mM NaOAc gradient in 100 mM NaOH) on CarboPac PA-1 of the oligosaccharide mixture generated from Suc + maltose (MOS DP2) with the GTFA enzyme at $t = 0$, 1, 3 and 6 h (pH 4.7, 37°C). Established oligosaccharide structures for isolated fractions are included. G = glucose; F = fructose. Two Fru-containing compounds are marked with an asterisk.

D_2O. Suppression of the HOD signal was achieved by applying a water eliminated Fourier transform pulse sequence for 1D experiments and by a pre-saturation of 1 s during the relaxation delay in 2D experiments. The 2D TOCSY spectra were recorded using an MLEV-17 (composite pulse devised by Levitt et al. (1982)) mixing sequence with spin-lock times of 20–200 ms. The 2D ROESY spectra were recorded using the standard Bruker XWINNMR software with a mixing time of 200 ms. The carrier frequency was set at the downfield edge of the spectrum in order to minimize TOCSY transfer during spin-locking. Natural abundance 2D ^{13}C–^{1}H HSQC experiments (^{1}H frequency 500.0821 MHz, ^{13}C frequency 125.7552 MHz) were recorded without decoupling during acquisition of the ^{1}H free induction decay. Resolution enhancement of the spectra was performed by a Lorentzian-to-Gaussian transformation for 1D spectra or by multiplication with a squared-bell function phase shifted by π/(2.3) for 2D spectra, and when necessary, a fifth-order polynomial baseline correction was performed. Chemical shifts (δ) are expressed in ppm by reference to internal acetone (δ 2.225 for ^{1}H and δ 31.07 for ^{13}C).

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest

None declared.

Abbreviations

DP, degree of polymerization; EPS, exopolysaccharide; Fru, fructose; GH70, glycoside hydrolase family 70; Glc, glucose; GTFA, glucosyltransferase; HPAEC, high-pH anion-exchange chromatography; HSQC, ^{1}H detected heteronuclear single quantum coherence spectroscopy; MALDI-TOF-MS, matrix-assisted laser-desorption ionization time-of-flight mass spectrometry; MOSs, malto-oligosaccharides; NMR, nuclear magnetic resonance; PAD, pulsed amperometric detection; ROESY, rotating-frame nuclear Overhauser enhancement spectroscopy; Suc, sucrose; TLC, thin-layer chromatography; TOCSY, total correlation spectroscopy.

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